

Production of fucosylated oligosaccharides in *Bacillus*

The present invention relates to the technical fields of genetic engineering, particularly to genetic engineering of *Bacillus* cells for the production of fucosylated oligosaccharides, and to the microbial production of fucosylated oligosaccharides using
5 said genetically engineered *Bacillus* cells.

Background

Human breast milk has optimal nutritional properties for infants. The saccharides that are present in human breast milk represent the main component of human milk, ahead of fats and proteins. In addition to lactose, which serves as an energy source,
10 human breast milk contains about 5 to 25 grams per liter of complex sugar molecules, i.e. oligosaccharides. These oligosaccharides are found at significant concentrations in only human milk, and they are collectively known as human milk oligosaccharides (HMOs).

Approximately 200 structurally distinct HMOs have been identified to date. Said
15 HMOs are based on the disaccharide lactose (consisting of a glucose (Glc) moiety and a galactose (Gal) moiety) and bear additional monosaccharide residues which are based on *N*-acetyl-glucosamine (GlcNAc), fucose (Fuc), sialic acid (NeuNAc), and galactose (Gal). The concentration and composition of HMOs in human milk varies between individuals and during the lactation period from up to 20 g/L in the
20 colostrum to 5-10 g/L in the mature milk.

Milk of women belonging to the so-called "secretor phenotype" contains a high content of α -1,2-fucosylated HMOs. These women express the *FUT2* gene encoding the so-called "fucosyltransferase 2". The most abundant HMOs in their milk are 2'-fucosyllactose (2'-FL; Fuc(α 1-2)Gal(β 1-4)Glc) and lacto-*N*-fucopentaose-I (LNPF-I;
25 Fuc(α 1-2)Gal(β 1-3)GlcNAc(β 1-3)Gal(β 1-4)Glc).

Human milk oligosaccharides are not digested during their transit through the intestine of infants. Due to their persistence in the infant's gut, they exhibit beneficial effects. More specifically, HMOs have been shown to be prebiotic as they serve as

carbon source for commensal microorganisms of the genera *Bifidobacterium*, *Bacteroides* and *Lactobacillus*. Therefore, HMOs support proliferation of these microorganisms in infants' guts.

Human milk oligosaccharides also directly reduce colonization of an infant's gut by pathogens in that they prevent adherence of said pathogens to glycan structures on the gut's mucosal surface. The HMOs function as a decoy due to their structural similarity to epithelial surface glycans and inhibit invasion of the pathogens thereby reducing the risk of infections.

Alpha-1,2-fucosylated HMOs have been shown to be protective against infections with *Campylobacter jejuni*, the causative agent of most common bacterial diarrheas. The α -1,2-fucosylated HMOs are also associated with protection against diarrhea caused by the heat stable toxin of *Escherichia coli*. Also, the risk of infections with diarrhea-mediating caliciviruses is reduced by a high content of α -1,2-fucosylated HMOs in breast milk. HMOs, especially the fucosylated HMO lacto-*N*-fucopentaose V (LNFP-V; Gal(β 1-3)GlcNAc (β 1-3)Gal(β 1-4)[Fuc α 1-3]Glc), bind(s) to the carbohydrate binding site of toxin A from *Clostridium difficile*, the most common cause of nosocomial diarrhea. Thus, HMOs seem to prevent the interaction of toxin A from *C. difficile* with cellular receptors.

Furthermore, adherence of *Pseudomonas aeruginosa* to epithelial cells was significantly inhibited by 2'-FL and 3-fucosyllactose (3-FL; Gal(β 1-4)[Fuc α 1-3]Glc). Binding of noroviruses (Norwalk-like viruses, NLV), the main cause of acute gastroenteritis, to histo-blood group antigens is prevented by α -1,2-fucosylated HMOs as well as by α -1,3-fucosylated HMOs. This indicates the potential of these HMOs to inhibit norovirus capsid-binding to host receptor glycans.

Due to the known benefits of HMOs, and especially of fucosylated HMOs, an economically worthwhile process for their synthesis is desired such that these oligosaccharides or at least some of these oligosaccharides become available as supplement for infant formula.

Due to the limited supply and difficulties of obtaining pure fractions of individual human milk oligosaccharides, chemical routes to the synthesis of some of these

complex molecules were developed. However, neither chemical synthesis nor biocatalytic approaches proved to be commercially sustainable. Moreover, particularly chemical synthesis of human milk oligosaccharides involves the use of several noxious chemicals, which impose the risk to contaminate the final product.

- 5 Due to the challenges involved in the chemical synthesis of human milk oligo-
saccharides, fermentative approaches for producing HMOs were developed. Today,
microbial production of several HMOs such as 2'-fucosyllactose, 3-fucosyllactose,
lacto-*N*-tetraose, lacto-*N*-neotetraose, lacto-*N*-fucopentaose I, lacto-*N*-difucohexa-
ose II, 3'-sialyllactose and 6'-sialyllactose using mainly genetically engineered
10 *Escherichia coli* strains have been disclosed.

- To date, recombinant *E. coli* cells are used for microbial production of HMOs in
industrial scale. However, the genus *Escherichia coli* comprises pathogenic
members as well as non-pathogenic members. Notwithstanding that non-pathogenic
E. coli strains are employed for the microbial production of HMOs, such non-
15 pathogenic *E. coli* are not recognized as safe for the manufacturing of products that
are intended for human consumption in a variety of jurisdictions. Therefore,
microbial cells of genera that are recognized as safe in such jurisdictions are
needed for the manufacturing of compounds that are intended for human consump-
tion to obtain regulatory approval of said products for their commercialization in said
20 jurisdictions.

The problem is solved by using bacterial cells of the genus *Bacillus* which are
recognized as being safe for human consumption, and which are capable of
producing a fucosylated oligosaccharide.

- Bacteria of the genus *Bacillus* are gram-positive, rod-shaped, endospore-forming
25 microbial cells of either aerobic or facultatively anaerobic species. The genus
Bacillus belongs to the phylum Firmicutes. The genome of the members of the
genus *Bacillus* possesses a bias towards A-T base pairs in its codon usage. *Bacillus*
species are almost ubiquitously present in nature. They can for example be found in
soil (*B. subtilis*), but also occur in extreme environments such as high pH (*B.*
30 *alcalophilus*), high temperature (*B. thermophilus*), or high salt (*B. halodurans*).

The genus *Bacillus* includes 266 named species which include free living species as well as parasitic pathogenic species. Two *Bacillus* species are considered of being medically significant: *B. anthracis*, which causes anthrax, and *B. cereus*, which causes food poisoning. A third species, *B. thuringiensis*, is an important insect pathogen producing a toxin that can kill insects. Thus, it is used as an insecticide to control insect pests.

Because of their GRAS (Generally Recognized as Safe) status, several *Bacillus* species, e.g. *B. amyloliquefaciens*, *B. licheniformis* and *B. subtilis*, are used in the biotechnological production of various proteins and compounds to be used in food and pharmaceutical industry.

B. amyloliquefaciens is the source of restriction enzyme BamHI, and also synthesizes the natural antibiotic protein barnase. In addition, *B. amyloliquefaciens* produces plantazolicin, an antibiotic with selective activity against *B. anthracis*. Alpha-amylase from *B. amyloliquefaciens* is often used in starch hydrolysis. *B. amyloliquefaciens* is also a source of subtilisin, which catalyzes the breakdown of proteins.

B. amyloliquefaciens is a root-colonizing bacterium that is used to fight some plant root pathogens in agriculture, aquaculture and hydroponics as it acts against bacterial and fungal pathogens, and may prevent infection by competitive exclusion or out-competing the unwanted pathogen.

Its high capacity of secreting alkaline serine protease has made *B. licheniformis* one of the most important bacteria in industrial enzyme production. Subtilisin Carlsberg secreted by *B. licheniformis* is used as a detergent protease, and is sold under the trade name Alcalase®.

B. subtilis is a catalase positive bacterium that is found in soil and the gastrointestinal tract of ruminants and humans. *B. subtilis* and substances derived from this endotoxin-free bacterium have been evaluated by different authoritative bodies for their safe and beneficial use in food. In the United States carbohydrase and protease enzymes from *B. subtilis* are recognized as generally safe (GRAS) by the US Food and Safety Administration (FDA). *Bacillus subtilis* has also been granted the "Qualified Presumption of Safety" status by the European Food Safety Authority.

Moreover, nontoxigenic and nonpathogenic strains of *B. subtilis* strain were commonly used in food. For example, fermented soy beans in the form of Natto, which is commonly consumed in Japan, contains as many as 10^8 viable *B. subtilis* cells per gram. The fermented beans are recognized for their contribution to a healthy gut flora and vitamin K₂ intake. The natto product and the *B. subtilis* var. *natto* as its principal component are FOSHU (Foods for Specified Health Use) approved by the Japanese Ministry of Health, Labor and Welfare as effective for preservation of health.

B. subtilis is easy to handle, grows rapidly and the culture conditions are simple. Recombinant *B. subtilis* strains are used in the production of polyhydroxyalkanoates, hyaluronic acid and various enzymes such as amylase and proteases.

Wild-type natural isolates of *B. subtilis* are difficult to work with as compared to laboratory strains that have undergone domestication processes of mutagenesis and selection. These domesticated strains often have improved capabilities to undergo natural competence development (uptake and integration of environmental DNA), growth, and loss of abilities needed "in the wild". In *B. subtilis*, linear DNA as well as multimeric forms of plasmid DNA are actively taken up by natural competent cells.

Under certain physiological conditions, a small subpopulation of *B. subtilis* cells becomes competent. In *B. subtilis*, natural competence is regulated by a complex regulatory network. Key regulators in this network are, amongst others, the master regulator of competence ComK and the transcriptional master regulator of sporulation Spo0A. Transformation efficiency of *B. subtilis* cells and probably the efficacy for integrating DNA into their genome can be improved by genetic engineering. This can be achieved by ectopic integration of an expression cassette, comprising a regulated promoter (e.g. the mannitol-inducible P_{mtlA} promoter) and the genes *comK* and *comS*, into the genome of *B. subtilis*. Additionally, this strategy allows transformation of *B. subtilis* by natural competence using a complex medium (e.g. LB).

For the production of fucosylated saccharides, *B. subtilis* can be genetically modified by various methods.

Gene integration and/or (simultaneous) gene inactivation by disruption or deletion can be achieved by homologous recombination. For an efficient homologous recombination, at least 400-500 bp of homology arms are necessary in *B. subtilis*.

Another method for targeted genome engineering is the modern CRISPR-Cas9
5 system. This rapid and markerless genome-editing tool can be used for large scale genomic deletions, small and large DNA insertions, gene silencing by introduction of a stop codon as well as introduction of point mutations. No previous genome modifications are required for the scarless genome editing by CRISPR-Cas9.

Random chromosomal integration of genes and insertional mutagenesis can be
10 performed using a modified mariner-derived transposon. This system is not biased toward hotspots in *B. subtilis* while showing high efficiency in random ectopic integration.

Although *Bacillus* species are used for industrial scale production of enzymes,
Bacillus cells have not been implemented to date for industrial scale production of
15 oligosaccharides, in particular of fucosylated oligosaccharides.

Chinese patent application CN 108 410 787 A discloses recombinant *Bacillus subtilis* cells which synthesizes lactyl-N-neotetraose. Said recombinant *B. subtilis* cells possess a lactose permease gene which has been integrated into the cell's genome. In addition, said *Bacillus* cell which bears a plasmid comprising a beta-1,3-
20 N-glucosamine transferase gene and a β -1,4-galactosyltransferase gene. The *B. subtilis* cells may be cultivated in the presence of exogenous lactose, and synthesizes lactyl-N-neotetraose at titers of up to about 1 g/L which is too little for economically reasonable industrial scale production.

Although a variety of patent applications mention *Bacillus* as a genus which is
25 suitable for the production of oligosaccharides such as lacto-N-neotetraose, no commercial use of *Bacillus* for the productions of fucosylated oligosaccharides, in particular of fucosylated human milk oligosaccharides has been implemented yet, presumably because of the significant efforts in metabolic engineering that are required to implement the necessary biosynthetic pathways for HMO production in
30 *Bacillus*. Whereas above-referenced *B. subtilis* for the production of LNnT relies on

donor substrates that are naturally occurring in *B. subtilis* cells, production of a fucosylated oligosaccharide in *Bacillus* requires implementation of a heterologous metabolic pathway in the cell for providing the required donor substrate GDP-fucose.

- 5 Chinese patent application CN 109 735 479 A discloses recombinant *Bacillus subtilis* cells for synthesizing 2'-fucosyllactose, wherein the expression of a lactose transport enzyme is enhanced, and which cell expresses a fucose kinase, a phosphate guanine transferase and a fucosyltransferase. The yield of 2'-fucosyllactose in the fermentation medium was reported to be between 0.424 g/L and 1.042 g/L.
- 10 It was nonetheless an object of the present invention to provide a bacterial cell of the genus *Bacillus* for the production of fucosylated oligosaccharides.

The object has been achieved by providing a *Bacillus* cell that possesses a lactose permease for import of exogenous lactose into the cell, a *de novo* GDP-fucose biosynthesis pathway for providing GDP-fucose, and a fucosyltransferase for

15 transfer of a fucose moiety from GDP-fucose to lactose. Said *Bacillus* cell can be cultivated in the presence of exogenous lactose to produce a fucosylated oligosaccharide.

Summary

- According to a first aspect, provided is a non-sporulating bacterial cell of the genus
- 20 *Bacillus* for the production of a fucosylated oligosaccharide, wherein the *Bacillus* cell possesses a lactose permease, a GDP-fucose biosynthesis pathway, and a fucosyltransferase.

- According to a second aspect, provided is the use of the non-sporulating bacterial cell of the genus *Bacillus* according to the first aspect for the production of a
- 25 fucosylated oligosaccharide.

According to a third aspect, provided is a method for the production of a fucosylated oligosaccharide, the method comprising:

- providing a non-sporulating bacterial cell of the genus *Bacillus*, wherein said *Bacillus* cell possesses a lactose permease, a GDP-fucose biosynthetic pathway, and a lactose-accepting fucosyltransferase;
- cultivating the *Bacillus* cell in a culture medium containing lactose and under conditions that are permissive for the *Bacillus* cell to produce the fucosylated oligosaccharide, and
- optionally retrieving the fucosylated oligosaccharide from the culture medium and/or the *Bacillus* cell.

According to a fourth aspect, provided are fucosylated oligosaccharides that were produced by a *Bacillus* cell which possesses a lactose permease, a GDP-fucose biosynthesis pathway and a fucosyltransferase.

According to a fifth aspect, provided is the use of a fucosylated oligosaccharide that was produced by a *Bacillus* cell which possesses a lactose permease, a GDP-fucose biosynthesis pathway and a fucosyltransferase for the manufacturing of a nutritional composition.

According to a sixth aspect, provided are nutritional compositions containing at least one fucosylated oligosaccharide that had been produced by a *Bacillus* cell which possesses a lactose permease, a GDP-fucose biosynthesis pathway and a fucosyltransferase.

Detailed description

The present invention concerns *Bacillus* cells for the production of fucosylated oligosaccharides, their use and methods for producing fucosylated oligosaccharides by cultivating said *Bacillus* cells in a culture medium containing lactose, and under conditions that are permissive for the production of said fucosylated oligosaccharide by said *Bacillus* cell.

For being able to produce a fucosylated oligosaccharide, a *Bacillus* cell has to provide a donor substrate comprising a fucose moiety, and an acceptor substrate being a disaccharide or an oligosaccharide to a fucosyltransferase such that the fucosyltransferase can transfer the fucose moiety from the donor substrate to said
5 acceptor substrate thereby generating the fucosylated oligosaccharide.

It is to be understood that the fucosylated oligosaccharide, said *Bacillus* cell is intended to produce, is the desired fucosylated oligosaccharide, whereas other fucosylated oligosaccharides that may be generated due to the promiscuity of the fucosyltransferase during production of the desired fucosylated oligosaccharide are
10 considered as undesired fucosylated oligosaccharides or by-products. A suitable donor substrate is GDP-L-fucose, and a suitable acceptor substrate for generating a fucosyllactose is the disaccharide lactose. The resulting desired fucosylated oligosaccharide is 2'-fucosyllactose, 3-fucosyllactose or 2',3-difucosyllactose.

Wild-type *Bacillus* cells neither synthesize lactose intracellularly nor do they
15 internalize and metabolize exogenous lactose. However, lactose is the acceptor substrate for a fucose moiety by a lactose-accepting fucosyltransferase in the formation of some fucosylated oligosaccharides. Hence, to be able to produce a fucosylated oligosaccharide such as 2'-fucosyllactose, 3-fucosyllactose or 2',3-difucosyllactose, a *Bacillus* cell has to have the capability of providing lactose to the
20 lactose-accepting fucosyltransferase, either by generating lactose intracellularly and/or by internalizing exogenous lactose.

In an embodiment, the *Bacillus* cell for producing a fucosylated oligosaccharide is able to internalize exogenous lactose by possessing a lactose permease. The term "exogenous" with respect to lactose as used herein refers to lactose that does not
25 originate from the *Bacillus* cell, i.e. being intracellularly synthesized by the *Bacillus* cell, but which originates from outside the *Bacillus* cell and is added to the culture medium in which the *Bacillus* cell is grown to produce the fucosylated oligosaccharide.

In an additional and/or alternative embodiment, the *Bacillus* cell has been genetically
30 engineered to be able to internalize exogenous lactose, i.e. to possess a

lactose permease. Thus, the *Bacillus* cell for producing a fucosylated oligo-saccharide possesses a heterologous lactose permease. A suitable lactose permease is *E. coli* LacY or a functional variant thereof.

- 5 The term "heterologous" as used herein with respect to proteins, polypeptides, enzymes and transporters, but also with respect to nucleic acid molecules and/or nucleotide sequences, refers to a molecule that is non-native to the species of cell which contains said molecule. The term "non-native" indicates that said molecule is not present in the naturally occurring or wild-type progenitor cell, i.e. the cell of the same species that is most commonly occurring in nature, of the *Bacillus* cell.
- 10 The term "functional variant" as used herein with respect to enzymes and/or transport molecules refers to proteins or polypeptides possessing the same activity (enzymatic, catalytic or translocating) as the referenced enzyme or transporter, but which has a different amino acid sequence than the referenced enzyme or transporter molecule. Thus, a typical variant of a protein/polypeptide differs in amino acid
- 15 sequence from the reference protein/polypeptide. A variant and the reference protein/polypeptide may differ in amino acid sequence by one or more substitutions, additions, and/or deletions in any combination. Hence, the term "functional variant" comprises truncated versions of the referenced protein/polypeptide which possesses the same activity as the reference protein/polypeptide. A substituted or inserted
- 20 amino acid residue may or may not be one encoded by the genetic code. A variant of a protein/ polypeptide may be a naturally occurring such as an allelic variant, or it may be a variant that is not known to occur naturally. Non-naturally occurring variants of proteins/polypeptides may be made by mutagenesis techniques, by direct synthesis, and by other recombinant methods known to the persons skilled in
- 25 the art. Within the scope of the present disclosure, also proteins and interspecies homologs are comprised by the term "variant", that have an amino acid sequence/ nucleic acid sequence that has greater than about 60% amino acid sequence identity, 65%, 70%, 75%, 80%, 85%, 90%, preferably 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or greater amino acid sequence identity, preferably over a
- 30 region of at least about 25, 50, 100, 200, 500, 1000, or more amino acids, to the referenced polypeptide.

The term "same activity" as used herein refers to an enzymatic, catalytic or transporting activity of a protein/polypeptide in qualitative manner only. Hence, "functional variant" also comprises variants which possess an increased or decreased activity as compared to the activity of the referenced protein/polypeptide.

- 5 In an additional and/or alternative embodiment, the *Bacillus* cell has been genetically engineered to contain and express a nucleotide sequence which encodes a lactose permease, preferably a nucleotide sequence that encodes the *E. coli* lactose permease LacY or a functional variant thereof.

The *E. coli* lactose permease LacY is encoded by the protein coding region (i.e. the
10 open reading frame) of the *E. coli lacY* gene (Gen Bank accession number: NP_414877.1). Hence, in an embodiment, the *Bacillus* cell has been genetically engineered to contain and express a nucleotide sequence that encodes the *E. coli* lactose permease LacY or a functional variant thereof.

In an additional and/or alternative embodiment, the codon usage of the nucleotide
15 sequence encoding the heterologous lactose permease is adapted to the codon usage of *Bacillus*. The codon usage of *B. subtilis* for example is peculiar in that the overall GC-content is below about 45 %, the GC-content of the first letter of the codons above about 51 %, the CG-content of the second letter of the codons below about 36.1 %, and the CG-content of the third letter of the codons below about
20 46 %.

For expression of the lactose permease, the *Bacillus* cell contains a recombinant lactose permease gene, wherein the nucleotide sequence which encodes the lactose permease is operably linked to expression control sequences which mediate expression of the lactose permease open reading frame.

- 25 The term "operably linked" as used herein, shall mean a functional linkage between a nucleic acid/gene expression control sequence (such as a promoter, signal sequence, or array of transcription factor binding sites) and a second nucleic acid sequence (typically referred to as "protein coding region", "open reading frame", and sometimes even as "gene"), wherein the expression control sequence effects
30 transcription and/or translation of the nucleotide sequence corresponding to the

second sequence. Accordingly, the term "promoter" designates DNA sequences which usually "precede" an open reading frame in a DNA polymer and provide a site for initiation of the transcription into mRNA. "Regulator" DNA sequences, also usually "upstream" of (i.e., preceding) an open reading frame in a given DNA
5 polymer, bind proteins that determine the frequency (or rate) of transcriptional initiation. Collectively referred to as "promoter/regulator" or "control" DNA sequence, these sequences which precede a selected open reading frame (or series of open reading frames) in a functional DNA polymer cooperate to determine whether the transcription (and eventual expression) of an open reading frame will occur. DNA
10 sequences which "follow" a gene in a DNA polymer and provide a signal for termination of the transcription into mRNA are referred to as transcription "terminator" sequences.

The recombinant lactose permease gene may be integrated into the *Bacillus* chromosome, or present as an episomal version on a plasmid within the *Bacillus* cell.

15 Expression of the heterologous lactose permease gene in the *Bacillus* cell enables the resulting *Bacillus* cell to internalize exogenously supplied lactose from the culture medium. The internalized lactose can then serve as an acceptor substrate for a fucosyltransferase (see herein below).

The *Bacillus* cell for producing a fucosylated oligosaccharide has to be able to
20 provide a donor substrate for transferring a fucose moiety to an acceptor substrate. The donor substrate for a fucose moiety is GDP-fucose. Hence, the *Bacillus* cell has to be able to intracellularly produce GDP-fucose. For intracellular biosynthesis of GDP-fucose, the *Bacillus* cell possesses a GDP-fucose biosynthesis pathway. The *Bacillus* has been genetically engineered to possess a GDP-fucose biosynthesis
25 pathway. The GDP-fucose biosynthesis pathway may either be a *de novo* GDP-fucose biosynthesis pathway and/or a GDP-fucose salvage pathway.

In an embodiment, the *Bacillus* cell possesses a *de novo* GDP-fucose biosynthesis pathway for intracellular biosynthesis of GDP-fucose. The *Bacillus* cell has been genetically engineered to possess the *de novo* GDP-fucose biosynthesis pathway.

The *de novo* GDP-fucose biosynthesis pathway comprises activities of the following enzymes:

1. a fructose-6-phosphate isomerase;
2. a phosphomannomutase;
- 5 3. a GDP:mannose-1-phosphate guanylyltransferase;
4. a GDP-mannose-4,6-dehydratase; and
5. a GDP-fucose synthase.

Hence, the *Bacillus* cell for producing a fucosylated oligosaccharide possesses a fructose-6-phosphate isomerase (typically also designated as mannose-6-phosphate isomerase), a phosphomannomutase, a GDP:mannose-1-phosphate guanylyltransferase, a GDP-mannose-4,6-dehydratase, and a GDP-fucose synthase.

In an additional and/or alternative embodiment, the *Bacillus* cell has been genetically engineered to possess a fructose-6-phosphate isomerase, a phosphomannomutase, a GDP:mannose-1-phosphate guanylyltransferase, a GDP-mannose-4,6-dehydratase, and a GDP-fucose synthase.

The *de novo* pathway of GDP-fucose biosynthesis starts from isomerization of fructose-6-phosphate to mannose-6-phosphate, a reaction that is catalyzed by the fructose-6-phosphate isomerase. In an additional and/or alternative embodiment, the *Bacillus* cell has been genetically engineered to contain and express a nucleotide sequence which encodes a fructose-6-phosphate isomerase. A suitable fructose-6-phosphate is *E. coli* ManA or a functional variant thereof. An exemplary nucleotide sequence is the nucleotide sequence which encodes *E. coli* ManA.

The *E. coli* fructose-6-phosphate isomerase ManA is encoded by the protein coding region of the *E. coli* *manA* gene (GenBank accession number: NP_416130.3). Thus, in an additional embodiment, the *Bacillus* cell has been genetically engineered to contain and express a nucleotide sequence which encodes the *E. coli* ManA or a functional variant thereof.

The *E. coli* fructose-6-phosphate isomerase ManA is encoded by the open reading frame of the *E. coli* *manA* gene (Gen Bank accession number: NP_416130.3). Thus,

in an additional and/or alternative embodiment, the *Bacillus* cell contains the *E. coli* *manA* gene or a functional variant thereof.

In an additional and/or alternative embodiment, the codon usage of the nucleotide sequence encoding the fructose-6-phosphate isomerase is adapted to the codon
5 usage of *Bacillus*.

Other suitable fructose-6-phosphate isomerases are *B. subtilis* ManA and the paralogous proteins *B. subtilis* GmuF (YdhS) and *B. subtilis* Pmi (Yvyl) or functional variants thereof. Exemplary nucleotide sequences are the nucleotide sequences which encodes *B. subtilis* ManA, *B. subtilis* GmuF or *B. subtilis* Pmi. The *B. subtilis*
10 fructose-6-phosphate isomerase ManA is encoded by the open reading frame of the *B. subtilis* *manA* gene (Gen Bank accession number: NP_389084.1).

The *B. subtilis* fructose-6-phosphate isomerase ManA is encoded by the protein coding region of the *B. subtilis* *manA* gene (GenBank accession number: (NP_389084.1). Thus, in an additional embodiment, the *Bacillus* cell has been
15 genetically engineered to contain and express a nucleotide sequence which encodes the *B. subtilis* ManA or a functional variant thereof. Thus, in an additional and/or alternative embodiment, the *Bacillus* cell contains the *B. subtilis* *manA* gene or a functional variant thereof. In another additional and/or alternative embodiment, the *Bacillus* cell has been genetically engineered to contain and express a nucleotide
20 sequence which encoded the *B. subtilis* *gmuF* gene or a functional variant thereof and/or a nucleotide sequence which encodes the *B. subtilis* *pmi* gene or a functional variant thereof.

In an additional and/or alternative embodiment, the expression of the native *manA* gene, the native *gmuF* gene and/or the native *pmi* gene can be increased. The
25 expression of the native *manA* gene, the native *gmuF* gene and/or the native *pmi* gene can be increased in that the endogenous promoter of at least one of the native *manA* gene, the native *gmuF* gene and the native *pmi* gene is replaced by a stronger promoter, i.e. a promoter mediating an increased expression as compared to the native promoter of the *manA* gene, the *gmuF* gene and the *pmi* gene
30 respectively. In another additional or alternative embodiment, the expression of the native *manA* gene, the native *gmuF* gene and/or the native *pmi* gene can be

enhanced in that additional copies of the native *manA* gene, the native *gmuF* gene and/or the native *pmi* gene are propagated in the *Bacillus* cell.

In another additional or alternative embodiment, the expression of the native *manA* gene can be enhanced by deletion or functional inactivation of the *manP* gene
5 encoding a mannose transporter leading to a constitutive expression of the native *manA* gene.

In another additional or alternative embodiment, the expression of the native *gmuF* gene can be enhanced by deletion or functional inactivation of the *gmuR* gene encoding the corresponding transcriptional repressor.

10 For expression of the fructose-6-phosphate isomerase, the *Bacillus* cell contains a recombinant fructose-6-phosphate isomerase gene, wherein the nucleotide sequence which encodes the fructose-6-phosphate isomerase is operably linked to expression control sequences.

The recombinant fructose-6-phosphate isomerase gene may be integrated into the
15 *Bacillus* chromosome or present as an episomal version on a plasmid within the *Bacillus* cell.

In the second step of the *de novo* GDP-fucose biosynthesis, mannose-6-phosphate is converted to mannose-1-phosphate by the enzymatic activity of a phosphomannomutase. In an additional and/or alternative embodiment, the *Bacillus* cell has been
20 genetically engineered to contain and express a nucleotide sequence which encodes a phosphomannomutase.

A suitable phosphomannomutase is the *E. coli* phosphomannomutase ManB. Thus, in an additional embodiment, the *Bacillus* cell has been genetically engineered to contain and express a nucleotide sequence which encodes *E. coli* ManB or a
25 functional variant thereof.

The *E. coli* phosphomannomutase ManB is encoded by the nucleotide sequence of the protein coding region of the *E. coli manB* gene (Gen Bank accession number:

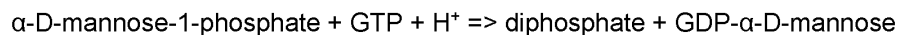
NP_416552.1). Thus, in an additional and/or alternative embodiment, the *Bacillus* cell contains the *E. coli manB* gene or a functional variant thereof.

In an additional and/or alternative embodiment, the codon usage of the nucleotide sequence encoding the phosphomannomutase is adapted to the codon usage of
5 *Bacillus*.

For expression of the phosphomannomutase, the *Bacillus* cell contains a recombinant phosphomannomutase gene, wherein the nucleotide sequence which encodes the phosphomannomutase is operably linked to expression control sequences.

10 The recombinant phosphomannomutase gene may be integrated into the *Bacillus* chromosome or present in the *Bacillus* cell as an episomal version on a plasmid.

The next step in the *de novo* biosynthesis pathway of GDP-fucose is the formation of GDP-mannose by the following reaction:



15 The conversion of mannose-1-phosphate to GDP-mannose is mediated by a GDP:mannose-1-phosphate guanylyltransferase. Thus, the *Bacillus* cell for producing a fucosylated oligosaccharide possesses a heterologous GDP:mannose-1-phosphate guanylyltransferase. A suitable GDP:mannose-1-phosphate guanylyltransferase is *E. coli* ManC or a functional variant thereof.

20 In an additional and/or alternative, the *Bacillus* cell has been genetically engineered to contain and express a nucleotide sequence which encodes a GDP:mannose-1-phosphate guanylyltransferase. An exemplary nucleotide sequence is the nucleotide sequence which encodes *E. coli* ManC. Thus, in an additional embodiment, the *Bacillus* cell has been genetically engineered to contain and express a nucleotide
25 sequence which encodes the *E. coli* ManC or a functional variant thereof.

The *E. coli* GDP:mannose-1-phosphate guanylyltransferase ManC is encoded by the open reading frame of the *E. coli manC* gene (Gen Bank accession number: NP_416553.1). Thus, in an additional and/or alternative embodiment, the *Bacillus* cell contains the *E. coli manC* gene or a functional variant thereof.

- 5 In an additional and/or alternative embodiment, the codon usage of the nucleotide sequence encoding the GDP:mannose-1-phosphate guanylyltransferase is adapted to the codon usage of *Bacillus*.

For expression of the GDP:mannose-1-phosphate guanylyltransferase, the *Bacillus* cell contains a recombinant GDP:mannose-1-phosphate guanylyltransferase gene,
10 wherein the nucleotide sequence which encodes the GDP:mannose-1-phosphate guanylyltransferase is operably linked to expression control sequences.

The recombinant GDP:mannose-1-phosphate guanylyltransferase gene may be integrated into the *Bacillus* chromosome or present as an episomal version on a plasmid.

- 15 In the subsequent step of the *de novo* GDP-fucose pathway, catalyzed by a GDP-mannose-4,6-dehydratase, GDP-D-mannose is converted to GDP-4-dehydro-6-deoxy-D-mannose.

Thus, the *Bacillus* cell for producing a fucosylated oligosaccharide possesses a GDP-mannose-4,6-dehydratase. A suitable GDP-mannose-4,6-dehydratase is *E.*
20 *coli* Gmd.

In an additional and/or alternative, the *Bacillus* cell has been genetically engineered to contain and express a nucleotide sequence which encodes a GDP-mannose-4,6-dehydratase. An exemplary nucleotide sequence is the nucleotide sequence which encodes *E. coli* Gmd. Thus, in an additional embodiment, the *Bacillus* cell has been
25 genetically engineered to contain and express a nucleotide sequence which encodes the *E. coli* Gmd or a functional variant thereof.

The *E. coli* GDP-mannose-4,6-dehydratase Gmd is encoded by the open reading frame of the *E. coli gmd* gene (Gen Bank accession number: NP_416557.1). Thus, in an additional and/or alternative embodiment, the *Bacillus* cell contains the *E. coli gmd* gene or a functional variant thereof.

- 5 In an additional and/or alternative embodiment, the codon usage of the nucleotide sequence encoding the GDP-mannose-4,6-dehydratase is adapted to the codon usage of *Bacillus*.

- For expression of the GDP-mannose-4,6-dehydratase, the *Bacillus* cell contains a recombinant GDP-mannose-4,6-dehydratase gene, wherein the nucleotide
10 sequence which encodes the GDP-mannose-4,6-dehydratase is operably linked to expression control sequences.

The recombinant GDP-mannose-4,6-dehydratase gene may be integrated into the *Bacillus* chromosome or present as an episomal version on a plasmid.

- In the final reaction of the *de novo* GDP-fucose biosynthesis pathway, GDP-4-
15 dehydro-6-deoxy-D-mannose is converted to GDP-fucose by a two-step NADPH-dependent reaction. This conversion is mediated by the GDP-fucose synthase and involves an epimerase reaction and a reductase reaction. Said epimerase reaction converts GDP-4-keto-6-deoxymannose to GDP-4-keto-6-deoxygalactose, which is then reduced to GDP-fucose.

- 20 Thus, the *Bacillus* cell for producing a fucosylated oligosaccharide possesses a GDP-fucose synthase. A suitable GDP-fucose synthase is *E. coli* WcaG or a functional variant thereof.

- In an additional and/or alternative, the *Bacillus* cell has been genetically engineered to contain and express a nucleotide sequence which encodes a GDP-fucose
25 synthase. An exemplary nucleotide sequence is the nucleotide sequence which encodes *E. coli* WcaG. Thus, in an additional embodiment, the *Bacillus* cell has been genetically engineered to contain and express a nucleotide sequence which encodes the *E. coli* WcaG or a functional variant thereof.

The *E. coli* GDP-fucose synthase WcaG is encoded by the open reading frame of the *E. coli* *wcaG* gene (Gen Bank accession number: NP_416556.1). Thus, in an additional and/or alternative embodiment, the *Bacillus* cell contains the *E. coli* *wcaG* gene or a functional variant thereof.

- 5 In an additional and/or alternative embodiment, the codon usage of the nucleotide sequence encoding the GDP-fucose synthase is adapted to the codon usage of *Bacillus*.

- For expression of the GDP-fucose synthase, the *Bacillus* cell contains a recombinant GDP-fucose synthase gene, wherein the nucleotide sequence which encodes the GDP-fucose synthase is operably linked to expression control sequences.
- 10

The recombinant GDP-fucose synthase gene may be integrated into the *Bacillus* chromosome or present as an episomal version on a plasmid.

- In an additional and/or alternative embodiment, the *Bacillus* cell possesses a GDP-fucose salvage pathway for the intracellular biosynthesis of GDP-fucose. In the salvage pathway of GDP-L-fucose, free cytosolic fucose is phosphorylated by L-fucokinase to form L-fucose-L-phosphate, which is then further converted to GDP-L-fucose.
- 15

The salvage pathway for biosynthesis of GDP-fucose comprises the following enzymes:

- 20 I. a fucose kinase; and
II. a L-fucose-1-phosphate guanylyltransferase.

Hence, the *Bacillus* cell for producing a fucosylated oligosaccharide which possesses a GDP-fucose salvage pathway possesses a fucose kinase and a L-fucose-1-phosphate guanylyltransferase.

- 25 In an additional and/or alternative embodiment, the *Bacillus* cell has been genetically engineered to possess a fucose kinase and a L-fucose-1-phosphate guanylyltransferase.

The fucose kinase, also called fucokinase, ATP:6-deoxy-L-galactose 1-phosphotransferase, ATP:β-L-fucose 1-phosphotransferase or L-fucokinase activity, L-fucose kinase activity catalyzes the reaction $\text{L-fucose} + \text{ATP} \rightarrow \beta\text{-L-fucose-1-phosphate} + \text{ADP} + 2 \text{H}^+$.

- 5 The L-fucose-1-phosphate guanylyltransferase or GDP-L-fucose pyrophosphorylase then converts said β-L-fucose-1-phosphate to GDP-L- fucose.

In an additional and/or alternative embodiment, the *Bacillus* cell has been genetically engineered to contain and express a nucleotide sequence which encodes a fucose kinase or a functional variant thereof, and a L-fucose-1-phosphate
10 guanylyltransferase or a functional variant thereof.

In an additional and/or alternative embodiment, the fucose kinase and the L-fucose-1-phosphate guanylyltransferase are combined in a single polypeptide. Suitable genes for transformation, coding for a fucose kinase, a fucose-1-phosphate guanylyltransferase and/or a bifunctional L-fucose-1-phosphate guanylyltransferase
15 can be obtained from the genera *Bacteroides*, *Lentisphaera*, *Ruminococcus*, *Solibacter*, *Arabidopsis*, *Oryza*, *Physcomitrella*, *Vitis*, *Danio*, *Bos*, *Equus*, *Macaca*, *Pan*, *Homo*, *Rattus*, *Mus* and *Xenopus*. An example for a bifunctional fucose kinase/L-fucose-1-phosphate guanylyltransferase is found in *Bacterioides fragilis*.

In *B. fragilis*, the bifunctional fucose kinase/L-fucose-1-phosphate guanylyltrans-
20 ferase Fkp is encoded by the *B. fragilis* fkp gene (GenBank accession number AY849806).

In an additional and/or alternative embodiment, the *Bacillus* cell has been genetically engineered to contain and express a nucleotide sequence that encodes the *B. fragilis* Fkp or a functional variant thereof.

25 In an additional and/or alternative embodiment, the codon usage of the nucleotide sequences encoding the fucose kinase, fucose-1-phosphateguanylyltransferase and/or the bifunctional fucose kinase/L-fucose-1-phosphate guanylyltransferase is adapted to the codon usage of *Bacillus*.

For expression of the fucose kinase and the fucose-1-phosphateguanylyltransferase, the *Bacillus* cell contains at least one recombinant gene, wherein the protein-coding region encoding the fucose kinase and the L-fucose-1-phosphate guanylyltransferase and/or the bifunctional fucose kinase/L-fucose-1-phosphate
5 guanylyltransferase fucose kinase is operably linked to expression control sequences.

The recombinant fucose kinase gene, fucose-1-phosphateguanylyltransferase gene and/or the bifunctional fucose kinase/L-fucose-1-phosphate guanylyltransferase gene may be integrated into the *Bacillus* chromosome or present as an episomal
10 version on a plasmid.

For the intracellular formation of fucosyllactose, the *Bacillus* cell possesses a fucosyltransferase. The enzymatic activity of the fucosyltransferase transfers the fucose moiety from the donor substrate to the acceptor substrate. For the biosynthesis of a fucosyllactose, said acceptor substrate is lactose. Hence, the fucosyl-
15 transferase is a lactose-accepting fucosyltransferase.

The fucosyltransferase is selected from the group consisting of α -1,2-fucosyltransferases for the biosynthesis of 2'-fucosyllactose, and α -1,3-fucosyltransferases for the biosynthesis of 3-fucosyllactose.

In an additional and/or alternative, the *Bacillus* cell has been genetically engineered
20 to contain and express at least one nucleotide sequence which encodes a fucosyltransferase.

For producing 2'-fucosyllactose (2'-FL), the α -1,2-fucosyltransferases WbgL from *E. coli* O126 and FucT2 from *Helicobacter pylori* (EP 2 479 263 B1), the α -1,2-fucosyltransferases WblA from *Vibrio cholera* O22, FutD from *H. bilis* ATCC 437879, FutE
25 from *H. cinaede* CCUG 18818, FutN from *Bacteroides vulgatus* ATCC 8482, FutO from *Bacteroides ovatus* ATCC 8483, WbgN from *E. coli* O55:H7, Bft1 and Bft3 from *Bacteroides fragilis* NCTC 9343 (WO 2014/018596 A2), and the α -1,2-fucosyltransferases FucT2 from *H. pylori* for the synthesis of Lewis Y and Lewis B saccharides

(US 6,670,160 B2) were described and are suitable α -1,2-fucosyltransferases for 2'-FL biosynthesis in *Bacillus* cells.

For producing 3-fucosyllactose, the α -1,3-fucosyltransferase Amuc from *Akkermansia muciniphila*, and FucT6 and FucT7 from *Bacteroides fragilis*
5 (EP 2 439 264 A1), the α -1,3-fucosyltransferase FutA from *H. pylori* (US 2014/0120611 A1) are described and are suitable α -1,3-fucosyltransferases for 3-FL biosynthesis in *Bacillus* cells. In addition, WO 2016/040531 A1 discloses the α -1,3-fucosyltransferase CafC from *B. nordii* CL02T12C05 for the synthesis of 3-fucosyllactose and lactodifucotetraose, and CafD from *H. hepaticus* ATCC51449 for the
10 production of LNnFP-III.

Additional fucosyltransferases that can be expressed in *Bacillus* cells for the production of fucosylated saccharides are disclosed in WO 2019/0088133 A1, which is incorporated herein by reference.

In an additional and/or alternative embodiment, the codon usage of the nucleotide
15 sequence encoding the fucosyltransferase is adapted to the codon usage of *Bacillus*.

For expression of the fucosyltransferase, the *Bacillus* cell contains at least one recombinant gene, wherein the protein-coding region encoding the fucosyltransferase is operably linked to expression control sequences.

20 The recombinant fucosyltransferase gene may be integrated into the *Bacillus* chromosome or present as an episomal version on a plasmid.

In an additional and/or alternative embodiment, the *Bacillus* cell for producing a fucosylated oligosaccharide is able to internalize L-fucose. The capability of internalizing L-fucose is of advantage for *Bacillus* cells which possess the GDP-
25 fucose salvage pathway.

In an additional and/or alternative embodiment, the *Bacillus* cell has been genetically engineered to enable or improve internalization of L-fucose. Thus, the

Bacillus cell possesses a heterologous L-fucose permease. A suitable L-fucose permease is *E. coli* FucP or a functional variant thereof.

In an additional and/or alternative embodiment, the *Bacillus* cell as been genetically engineered to contain and express a nucleotide sequence which encodes a L-fucose permease, preferably a nucleotide sequence that encodes the *E. coli* FucP or a functional variant thereof.

The *E. coli* L-fucose permease is encoded by the protein coding region of the *E. coli* *fucP* gene (Gen Bank accession number: NP_417281.1). Hence, the *Bacillus* cell has been genetically engineered to contain and express a nucleotide sequence that encodes the *E. coli* FucP or a functional variant thereof.

In an additional and/or alternative embodiment, the codon usage of the nucleotide sequence encoding the L-fucose permease is adapted to the codon usage of *Bacillus*.

For expression of the L-fucose permease, the *Bacillus* cell contains a recombinant L-fucose permease gene, wherein the nucleotide sequence which encodes the L-fucose permease is operably linked to expression control sequences.

The recombinant L-fucose permease gene may be integrated into the *Bacillus* chromosome or present as an episomal version on a plasmid.

In an additional and/or alternative embodiment, the *Bacillus* cell for producing a fucosylated oligosaccharide lacks any β -galactosidase activity or possesses reduced β -galactosidase activity as compared to a wild type *Bacillus* cell of the same species.

The intracellular biosynthesis of fucosylated oligosaccharides requires import of lactose as acceptor substrate for the lactose-accepting fucosyltransferase. Any intracellular enzyme activity that hydrolyzes internalized lactose would affect the efficacy of fucosyllactose formation as the pool of intracellular lactose would be diminished. Therefore, it would be advantageous if the *Bacillus* cell for producing a

fucosylated oligosaccharide would lack or at least possess – as compared to the wild-type *Bacillus* cell – reduced beta-galactosidase activity.

In an additional and/or alternative embodiment, the *Bacillus* cell has been genetically engineered to abolish or at least reduce the cell's β -galactosidase activity.

In an additional and/or alternative embodiment, the *Bacillus* cell has been genetically engineered by deletion or functional inactivation of the *ganA* gene. In another embodiment, the *Bacillus* cell has been genetically engineered to reduce the expression level of the *ganA* gene – as compared to the wild-type *Bacillus* cell.

10 The *Bacillus ganA* gene is also called *yvN* or *lacA*. It is a gene of the GanR regulon which contains genes encoding enzymes involved in the utilization of galactan. The *ganA* gene encodes a beta-galactosidase that is involved in the galactan utilization of *Bacillus*.

15 Deletion or functional inactivation of the *ganA* gene abolishes the GanA-mediated β -galactosidase activity in the *Bacillus* cell, whereas reduction of the *ganA* expression lowers the amount of GanA in the *Bacillus* cell and hence the β -galactosidase activity which could interfere with the biosynthesis of a fucosylated oligosaccharide.

In an additional and/or alternative embodiment, the *Bacillus* cell has been genetically engineered by deletion or functional inactivation of the *yesZ* gene. The *Bacillus yesZ* gene encodes the beta-galactosidase YesZ which plays a role in the degradation of rhamnogalacturonan being derived from plant cell walls. The expression of *Bacillus yesZ* gene is induced by rhamnogalacturonan I. In another embodiment, the *Bacillus* cell has been genetically engineered to reduce the expression level of the *yesZ* gene – as compared to the wild-type *Bacillus* cell.

25 Deletion or functional inactivation of the *yesZ* gene abolishes the YesZ-mediated β -galactosidase activity in the *Bacillus* cell, whereas reduction of the *yesZ* expression lowers the amount of YesZ in the *Bacillus* cell and hence the β -galactosidase activity which could interfere with the biosynthesis of a fucosylated oligosaccharide.

When *B. subtilis* enters the post-exponential growth phase, they (start to) produce large quantities of extracellular proteases. Foreign proteins are often protease sensitive. Therefore, an exoprotease-free strain is desirable to increase the stability of heterologous proteins and to allow accumulation of high levels of foreign proteins.

- 5 The genome of *Bacillus* encodes for at least eight extracellular proteases, namely *nprE*, *aprE*, *epr*, *bpr*, *mpr*, *nprB*, *vpr* and *wprA*. Thus, in an additional and/or alternative embodiment, the *Bacillus* cell has been genetically engineered in that at least one gene encoding for an extracellular protease has been deleted or functionally inactivated, preferably at least one of the genes selected from the group
- 10 consisting of *nprE*, *aprE*, *epr*, *bpr*, *mpr*, *nprB*, *vpr* and *wprA*. Preferably, two, three, four, five six, seven or eight of the genes selected from the group consisting of *nprE*, *aprE*, *epr*, *bpr*, *mpr*, *nprB*, *vpr* and *wprA* have been deleted or functionally inactivated.

- B. subtilis* synthesizes pulcherriminic acid when growing in media containing a
- 15 carbohydrate such as glucose or lactose. Excreted pulcherriminic acid forms the red pigment pulcherrimin, the salt of pulcherriminic acid (a ferric chelate), when iron is present in the growth medium. The formation of this undesired by-product during fermentation processes can be avoided/abolished by deletion or disruption of the genes *yvmC* and/or *cypX*. The gene *yvmC* encodes a cyclodipeptide synthase and
- 20 the gene *cypX* encodes a cytochrome P450 cyclo-l-leucyl-l-leucyl dipeptide oxidase.

Thus, in an additional and/or alternative embodiment, the *Bacillus* cell has been genetically engineered in that at least one of the genes *yvmC* and *cypX* has been deleted or functionally inactivated.

- The rhizobacterium *B. subtilis* possesses genes for the synthesis of more than 20
- 25 antibiotics. Among them are peptide antibiotics like *Bacillus subtilis* lantibiotics and lantibiotic-like peptides (subtilin, ericin S, mersacidin, sublancin 168, subtilosin A) and non-ribosomally synthesized (peptide) antibiotics (surfactin, iturin, bacillomycin, mycosubtilin, corynebactin/ bacillibactin, fengycin plipastatin, mycobacillin, TL-119, bacilysin, bacilysocin, amicoumacin, 3,3'-neotrehalosadamine, difficidin, rhizoctin).

For producing a fucosylated oligosaccharide, it is preferred to use a *Bacillus* cell that does not produce an antibiotic. Thus, in an additional and/or alternative embodiment, the *Bacillus* cell does not synthesize one or more of the antibiotics selected from the group consisting of lantibiotics and lantibiotic-like peptides such as subtilin, ericin S, mersacidin, sublancin 168, subtilosin A; non-ribosomally synthesized (peptide) antibiotics such as surfactin, iturin, bacillomycin, mycosubtilin, corynebactin/ bacillibactin, fengycin plipastatin, mycobacillin, TL-119, bacilysin, bacilysocin, amicoumacin, 3,3'-neotrehalosadiamine, difficidin and rhizocticin. The *Bacillus* cell may have been genetically engineered to obtain a *Bacillus* cell that does not synthesize one or more of said antibiotics.

Wild-type *Bacillus* cells are capable of forming spores. Sporulation, i.e. the process of forming spores, in bacteria is considered a reaction of the bacterial cell which initiates a developmental program leading to the formation of daughter cells of distinct morphology and fate. Sporulation of *Bacillus* was studied as a basic model for cell differentiation. During sporulation, a rod-shaped *Bacillus* cell divides asymmetrically, thereby resulting in two genetically identical cells with different morphology and fates.

However, in industrial production it is not desired if the bacterial production strain sporulates during fermentation. Therefore, it is preferred to use *Bacillus* cells for the production of fucosylated oligosaccharides that are not capable of forming spores. Such *Bacillus* cells are called "non-sporulating".

Preferably, the non-sporulating *Bacillus* cell capable to produce a fucosylated oligosaccharide originated from one of the *B. subtilis* strains listed in Table 1.

Strain	Manufacturer/Reference	Product	Application
<i>Bacillus subtilis</i> 168	BGSCID 1A1	-	reference strain
<i>Bacillus subtilis</i> 168 Δ spoII $GA::erm$	BGSCID BKE15310	-	reference strain
<i>Bacillus subtilis</i> 168 Δ sigE $::erm$	BGSCID BKE15320	-	reference strain
<i>Bacillus subtilis</i> 168 Δ sigG $::erm$	BGSCID BKE15330	-	reference strain
<i>Bacillus subtilis</i> 168 Δ sigF $::erm$	BGSCID BKE23450	-	reference strain
<i>Bacillus subtilis</i> 3NA	BGSCID 1S1	-	reference strain
<i>Bacillus subtilis</i> W23	BGSCID 2A9	-	reference strain
<i>Bacillus subtilis</i> PY79	BGSCID 1A747	-	reference strain
<i>Bacillus subtilis</i>	Garden of Life®	Primal Defense®	Human use
<i>Bacillus subtilis</i>	Roux-Ocefa Laboratorios	Totalflora	Human use
<i>Bacillus subtilis</i>	Ist. Bioch. Italiano	Lactipan Plus	Human use
<i>Bacillus subtilis</i> 2335	Biopharma	Biosporin®	Human use
LifeinU™ <i>Bacillus subtilis</i> CU1	Lesaffre Human Care	Super Smart®	Human use
<i>Bacillus subtilis</i> PXN/21	Probiotics International Ltd	Bio-Kult®	Human use
<i>Bacillus subtilis</i> HU58	Microbiome LABS	HU58™	Human use
<i>Bacillus subtilis</i> R0179	Hanmi Pharmaceutical Co. Ltd.	Medilac®	Human use
<i>Bacillus subtilis</i> DE111	Deerland Enzymes & Probiotics	NutriCommit	Human use
<i>Bacillus subtilis</i> DSM21097	MorinagaGarden B.V.	Morinaga Plus+	Human use
<i>Bacillus subtilis</i> IDCC1101	Ildong Pharmaceutical	Biobaby®	Human use
<i>Bacillus subtilis</i> KATMIRA1933	JSL Foods	YoguFarm	Human use
<i>Bacillus subtilis</i> spp. Natto	various	Natto	Human use
<i>Bacillus subtilis</i> SC-8	various	Cheonggukjang	Human use
<i>Bacillus subtilis</i> CH201/DSM5750	Christian Hansen	BioPlus2B®	Veterinary Use
<i>Bacillus subtilis</i> PB6	Kenim®	CLOSTAT®	Veterinary Use

Table 1: Non-limiting list of *Bacillus subtilis* strains.

In an additional and/or alternative embodiment, the *Bacillus* cell has been genetically engineered by deletion or functional inactivation of Spo0A. Suitable functional inactivation of Spo0A includes deletion of the phosphorylation site, where

5 Spo0F and Spo0B phosphotransferases phosphorylate Spo0A.

In an additional and/or alternative embodiment, the *Bacillus* cell has been genetically engineered by deletion or functional inactivation of the genes encoding sigma factor SigE (*sigE*) and/or sigma factor SigF (*sigF*).

The *Bacillus* cell of the invention is able to produce a fucosylated oligosaccharide when cultivated in the presence of lactose in a medium and under conditions that are permissive for the *Bacillus* cell to produce the fucosylated oligosaccharide.

- According to the second aspect, provided is the use of a *Bacillus* cell as described
5 herein before for the production of a fucosylated oligosaccharide, preferably a fucosylated human milk oligosaccharide such as 2'-FL, 3-FL or 2',3-DiFL.

- Since *Bacillus* is generally recognized as safe, production of fucosylated oligosaccharides for human consumption utilizing a production organism that is considered as generally safe should be considered safe too, provided that the
10 amendments to the *Bacillus* cells for producing the fucosylated oligosaccharide does not affect the safety of the production strain with respect to human health and the environment. Hence, it is expected that there will be less concerns affecting regulatory approval of a fucosylated oligosaccharide for human consumption, in particular of fucosylated human milk oligosaccharides for human consumption, more
15 specifically for infant formula, which consists of or contains a fucosylated oligosaccharide that has been produced by using a *Bacillus* cell as described herein. Thus, acceptance of microbially produced fucosylated oligosaccharides in infant formula and nutritional compositions at regulatory authorities as well as among consumers should be better.

- 20 According to the third aspect, provided is a method for the production of a fucosylated oligosaccharide the method comprises:
- providing a non-sporulating *Bacillus* cell that had been genetically engineered to possess a lactose permease, a GDP-fucose biosynthesis pathway, and a fucosyltransferase;
 - 25 - cultivating the *Bacillus* cell in a culture medium containing lactose and under conditions that are permissive for the production of the fucosylated oligosaccharide, and
 - optionally retrieving the fucosylated oligosaccharide from the culture medium and/or from the *Bacillus* cell.

The non-sporulating *Bacillus* cell that is provided is a *Bacillus* cell as described herein.

In an additional and/or alternative embodiment, the fucosylated oligosaccharide is selected from the group consisting of 2'-fucosyllactose (2'-FL), 3-fucosyllactose (3-FL), 2',3-difucosyllactose (DiFL), lacto-*N*-fucopentaose I (LNFP I), lacto-*N*-neofucopentaose I (LNnFP I), lacto-*N*-fucopentaose II (LNFP II), lacto-*N*-fucopentaose III (LNFP III), lacto-*N*-fucopentaose V (LNFP V), lacto-*N*-neofucopentaose V (LNnFP V), lacto-*N*-difucohexaose I (LNDH I) and lacto-*N*-difucohexaose II (LND).

In an additional and/or alternative embodiment, the culture medium contains L-fucose, in particular for cultivating a *Bacillus* cell for the production of a fucosylated oligosaccharide which possesses the GDP-fucose salvage pathway for providing GDP-fucose as donor substrate for the fucosyltransferase.

The invention also extends to fucosylated oligosaccharides that were produced by a *Bacillus* cell and/or a method as described herein, to the use of said fucosylated oligosaccharides for the manufacturing of a nutritional composition, preferably an infant formula, a dietary supplement or a medicinal food. Moreover, the present invention also extends to nutritional compositions containing a fucosylated oligosaccharide that was produced by a *Bacillus* cell and/or a method as described herein.

The present invention will be described with respect to particular embodiments and with reference to drawings, but the invention is not limited thereto but only by the claims. Furthermore, the terms first, second and the like in the description and in the claims, are used for distinguishing between similar elements and not necessarily for describing a sequence, either temporally, spatially, in ranking or in any other manner. It is to be understood that the terms so used are interchangeable under appropriate circumstances and that the embodiments of the invention described herein are capable of operation in other sequences than described or illustrated herein.

It is to be noticed that the term “comprising”, used in the claims, should not be interpreted as being restricted to the means listed thereafter; it does not exclude other elements or steps. It is thus to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude
5 the presence or addition of one or more other features, integers, steps or components, or groups thereof. Thus, the scope of the expression “a device comprising means A and B” should not be limited to devices consisting only of components A and B. It means that with respect to the present invention, the only relevant components of the device are A and B.

10 Reference throughout this specification to “one embodiment” or “an embodiment” means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, appearances of the phrases “in one embodiment” or “in an embodiment” in various places throughout this specification are not necessarily all
15 referring to the same embodiment, but may. Furthermore, the particular features, structures or characteristics may be combined in any suitable manner, as would be apparent to one of ordinary skill in the art from this disclosure, in one or more embodiments.

Similarly, it should be appreciated that in the description of exemplary embodiments
20 of the invention, various features of the invention are sometimes grouped together in a single embodiment, figure, or description thereof for the purpose of streamlining the disclosure and aiding in the understanding of one or more of the various inventive aspects. This method of disclosure, however, is not to be interpreted as reflecting an intention that the claimed invention requires more features than are
25 expressly recited in each claim. Rather, as the following claims reflect, inventive aspects lie in less than all features of a single foregoing disclosed embodiment. Thus, the claims following the detailed description are hereby expressly incorporated into this detailed description, with each claim standing on its own as a separate embodiment of this invention.

30 Furthermore, while some embodiments described herein include some but not other features included in other embodiments, combinations of features of different

embodiments are meant to be within the scope of the invention, and form different embodiments, as would be understood by those in the art. For example, in the following claims, any of the claimed embodiments can be used in any combination.

Furthermore, some of the embodiments are described herein as a method or
5 combination of elements of a method that can be implemented by a processor of a computer system or by other means of carrying out the function. Thus, a processor with the necessary instructions for carrying out such a method or element of a method forms a means for carrying out the method or element of a method.
Furthermore, an element described herein of an apparatus embodiment is an
10 example of a means for carrying out the function performed by the element for the purpose of carrying out the invention.

In the description and drawings provided herein, numerous specific details are set forth. However, it is understood that embodiments of the invention may be practiced without these specific details. In other instances, well-known methods, structures
15 and techniques have not been shown in detail in order not to obscure an understanding. The invention will now be described by a detailed description of several embodiments of the invention. It is clear that other embodiments of the invention can be configured according to the knowledge of persons skilled in the art without departing from the true spirit or technical teaching of the invention, the
20 invention being limited only by the terms of the appended claims.

Examples

Example 1: Transformation of *Bacillus subtilis*

Bacillus subtilis can be genetically manipulated by various techniques. For transformation of *B. subtilis*, competent cells were prepared by a modified protocol
25 of the two-step method (Anagnostopoulos, C. and Spizizen, J. (1961) J Bacteriol 81 (5): 741–746). An overnight culture was inoculated in MG1 medium and shaken at 37 °C. MG1 medium is Spizizen's minimal medium, which is supplemented with 0.5 % glucose, 5 mM MgSO₄ and 0.02 % casamino acids (optionally additionally supplemented with biotin and/or L-tryptophan). At the next morning, this culture was
30 diluted 1:20 in fresh MG1 medium and incubated at 37 °C for approximately 6 h.

- 1 ml of the culture was diluted in 8 ml MG2 medium which differs from MG1 medium in the concentration of casamino acids (0.01 % instead of 0.02 %). In a shortened protocol the overnight culture is directly diluted in MG2 medium. After incubation for another 90 min, a 1-ml portion of the culture was mixed with 1-3 µg multimeric
- 5 plasmid DNA or linear DNA and incubated at 37 °C for 30-60 min with shaking. Multimeric plasmid DNA was obtained either by using *E. coli* strain NM538 for propagation of plasmid DNA or by linearization of the plasmid by digestion with a single-cutter restriction enzyme, which cleaves within the backbone, followed by re-ligation with T4 DNA ligase.
- 10 Afterwards, cells were spread on 2x YT agar plates containing the appropriate antibiotic. Antibiotics were added in the following concentrations: 5 µg·mL⁻¹ erythromycin, 5 µg·mL⁻¹ chloramphenicol, 10 µg·mL⁻¹ kanamycin, 100 µg·mL⁻¹ spectinomycin.
- Alternatively, for protoplast transformation (Romero, D. et al. (2006) Journal of
- 15 Microbiological Methods 66:556-559), cells were grown in 20 ml of Penassay broth (PAB) at 37 °C until the onset of the stationary phase of growth ($OD_{600}=1.7-2$). Cells were then pelleted and resuspended in 10 ml of SMPP medium (0.3% bovine serum albumin, 5% 2 M sucrose, 25% 4x PAB, 50% 2x SMM), composition of 2x SMM being 1 M sucrose, 0.04 M maleic acid disodium salt hydrate and 0.04 M MgCl₂ (pH
- 20 6.5). After the addition of lysozyme (10 mg ml⁻¹) and mutanolysin (75 U ml⁻¹) the mixture was incubated at 37 °C with shaking to generate protoplasts. Protoplast formation was checked microscopically. Protoplasts were then carefully harvested by centrifugation at 5200 ×g and 4 °C for 5 min, washed twice with ice cold washing-electrotransformation buffer (1x SMM) and finally suspended in this solution.
- 25 Plasmid DNA (1-3 µg) was added to 120 µl of protoplast suspension and the mixture was kept on ice for at least 5 min. The transformation mixture was transferred to a 0.2-cm cuvette and a single electroporation pulse was applied at 25 µF, 400 Ω and 0.7 kV. Immediately after the electroporation shock 1 ml of recovering medium (equal volumes of 4x PAB and 2x SMM, prepared freshly before use) was added to
- 30 the cuvette. The transformation reaction was then transferred to a 2 ml tube and incubated at 37 °C with shaking for 12 h. For regeneration, the cell suspension was spread on DM3 agar plates (Chang, S. and Cohen, S. (1979) MGG 168(1):111-115)

and incubated at 37 °C for 48 h. DM3 regeneration medium contained the following sterile solutions per liter: 200 ml 4% agar, 100 ml 5% casamino acids, 50 ml 10% yeast extract, 100 ml 3.5% K₂HPO₄ and 1.5% KH₂PO₄, 25 ml 20% glucose, 20 ml 1 M MgCl₂, 500 ml 0.5 M sorbitol and 5 ml filter sterilized 2% bovine serum albumin
5 (added to the mixture when the temperature is below 55 °C) and was supplemented with the appropriate antibiotic.

Electroporation of *B. subtilis* was done according to a modified protocol from Zhang et al. (2011) provided by MoBiTec GmbH (Zhang, G., Bao, P., Zhang, Y., Deng, A., Chen, N. and Wen, T. (2011) Anal. Biochem., 409:130–137). A 2x YT overnight
10 culture was diluted 100-fold with fresh 2x YT medium and the culture was grown to an OD₆₀₀ of 0.2 at 37 °C on a rotary shaker. Then the culture was supplemented with 1% DL-threonine, 2% glycine, 0.1% tryptophan and 0.03% Tween 80. After cultivation for another 60 min, the cell suspension was cooled on ice for 20 min, centrifuged at 5000 x g for 10 min at 4 °C and washed twice with electroporation
15 buffer (0.5 M trehalose, 0.5 M sorbitol, 0.5 M mannitol, 0.5 mM MgCl₂, 0.5 mM K₂HPO₄, 0.5 mM KH₂PO₄, pH 7.4, filter-sterilized and stored frozen). Finally, cells were resuspended in electroporation buffer at 1/100 of the original culture volume and 100 µl of cell suspension was mixed with DNA. The transformation mixture was transferred to a 0.1-cm cuvette and electroporation was performed at 1.8 kV with a
20 single pulse delivered with a MicroPulser™ device (Bio-Rad). Immediately after pulse delivery, 1 ml 2x YT broth containing 0.5 M sorbitol and 0.38 M mannitol was added to the cuvette. The transformation suspension was transferred to a 2 ml tube and incubated at 37 °C for 3 h on a rotary shaker. The cells were spread on a selective 2x YT agar plate and incubated at 37 °C overnight.

25 Using an alternative electroporation protocol (Xue, G. P., J. S. Johnson, and B. P. Dalrymple: 1999; Journal of Microbiological Methods 34:183-191), 5 ml of LB containing 0.5 M glucitol was inoculated with *B. subtilis* and incubated overnight at 37 °C. Subsequently, the overnight culture was diluted (1:16) by 75 ml of LB containing 0.5 M glucitol and incubated until an OD₆₀₀ of 0.85 – 0.95 was obtained.
30 The cells were then pelleted by centrifugation for 10 min at 4 °C at 5.000xg and washed four times by ice-cooled electroporation buffer (10 % glycerol, 0.5 M glucitol, 0.5 M mannitol). Finally, the cells were resuspended in 1-2 ml electroporation buffer.

- Electroporation was performed using 60 μ l of competent cells with DNA in a cooled electroporation cuvette (1-mm electrode gap). The cell-DNA mixture was subjected to a single electrical pulse at 25 μ F, 200 Ω and 21 kV/cm. Finally, 1 ml recovery broth (LB containing 0.5 M glucitol and 0.38 mannitol) was added to the
- 5 electroporated cells and the bacterial culture was incubated for 3 h at 37 °C followed by plating on the LB agar supplemented by an antibiotic.

Two different rich media were used, namely Luria Broth (LB) and 2x YT.

Luria Broth (LB) medium consisted of 1% tryptone, 0.5% yeast extract and 0.5% NaCl (pH 7.2).

- 10 2x YT medium consisted of 1.6% tryptone, 1% yeast extract and 0.5% NaCl (pH 7.5).

To prepare rich-medium agar plates 15 g L⁻¹ agar were added.

For shaking flask experiments, Spizizen's minimal medium (Spizizen, J. 1958 Proc. Natl. Acad. Sci. U. S. A. 44(10):1072-1078) was used.

- 15 Spizizen's minimal medium contains the following salts: 2 g/L (NH₄)₂SO₄, 14 g/L K₂HPO₄, 6 g/L KH₂PO₄, 1 g/L Na₃ citrate $\times$ 2·H₂O and 0.2 g/L MgSO₄ $\times$ 7·H₂O.

The preculture medium consisted of Spizizen's minimal salts supplemented with 2 % D-glucose, 0.05 % casamino acids and MgSO₄ to a final concentration of 2 mM (optionally additionally supplemented with biotin and/or L-tryptophan).

- 20 The main culture medium consisted of Spizizen's minimal salts supplemented with 2 % D-glucose, 0.05 % casamino acids, MgSO₄ to a final concentration of 2 mM and 0.5 mL·L⁻¹ 1000x trace element solution (optionally additionally supplemented with biotin and/or L-tryptophan).

- Trace element solution (1000x) consisted of 100.6 g L⁻¹ C₆H₅NO₆, 56.4 g·L⁻¹ ammonium ferric citrate, 9.8 g·L⁻¹ MnCl₂ $\times$ 4·H₂O, 1.6 g·L⁻¹ CoCl₂ $\times$ 6·H₂O, 1 g·L⁻¹ CuCl₂ $\times$ 2·H₂O, 1.9 g·L⁻¹ H₃BO₃, 9 g·L⁻¹ ZnSO₄ $\times$ 7·H₂O, 1.1 g·L⁻¹ Na₂MoO₄ $\times$ 2·H₂O, 1.5 g·L⁻¹ Na₂SeO₃, 1.5 g·L⁻¹ NiSO₄ $\times$ 6·H₂O.
- 25

If necessary, the appropriate antibiotic(s) were added to the medium to make it selective.

B. subtilis strains were initially grown on rich-media agar plates to obtain single colonies. These plates were grown for 1 day at 30-37°C. For shaking flask experiments, a 20-ml preculture was inoculated with a single colony and grown over night at 30-37 °C on a rotary shaker. Subsequent 20-ml main cultures were inoculated with this preculture to a starting OD₆₀₀ of about 0.1 and incubated at 30-37 °C on a rotary shaker. If induction was required, a 40-60 ml main culture was splitted into 20-ml proportions at the timepoint of induction. The culture volume did not exceed 20 % of the shaking flask capacity.

Example 2: Construction of a *Bacillus subtilis* production strain for 2'-fucosyllactose

Metabolic engineering of a sporulation-deficient *Bacillus subtilis* strain (table 1) was achieved by the integration of the heterologous genes *E. coli manC*, *E. coli manB* and *E. coli manA* and simultaneous deletion of the endogenous gene *lacA* by homologous recombination. The *B. subtilis* gene *ganA* (*yvfN*, *lacA*), which is positioned within the galactan operon, encodes a beta-galactosidase.

For synthesis of GDP-mannose the open reading frames of *manC*, *manB* and *manA* were operably linked to the *B. subtilis* constitutive promoter P43 (iGem part repository: sequence ID: BBa_K143013) as an operon. The gene *manC* (Gen Bank accession number: NP_416553.1) encodes GDP:mannose-1-phosphate guanylyltransferase from *E. coli*. The gene *manB* (Gen Bank accession number: NP_416552.1) encodes *E. coli* phosphomannomutase and the gene *manA* (Gen Bank accession number: NP_389084.1) encodes *B. subtilis* fructose-6-phosphate isomerase. Each of the genes mentioned above was fused *in silico* to a *B. subtilis* RBS sequence. The herein described expression cassette <P43-*manCBA*> was codon-optimized for expression in *B. subtilis* and prepared synthetically by GenScript Corp. Subsequently, the complete integration cassette was assembled and cloned into pBR322 (New England Biolabs GmbH, Frankfurt, Germany) to create the suicide plasmid <pBR322 flank *ganA* up-*lox71-erm-lox66*-P43-*manCBA*-flank *ganA* down> (SEQ ID NO: 1). Afterwards, *B. subtilis* was transformed with this

plasmid by its natural competence. Cells were spread on 2x YT agar plates containing the appropriate antibiotic (5 µg mL⁻¹ erythromycin). Integration of the expression cassette <P43-*manCBA*> into the *ganA* locus of the *B. subtilis* genome, yielding strain A, was verified by colony PCR. Gene expression was confirmed by
5 targeted proteomics and/or by real-time PCR.

For the production of 2'-fucosyllactose from lactose and GDP-mannose in the resulting <P43-*manCBA*> integration strain, an expression plasmid (SEQ ID NO: 2) was constructed that comprises all necessary genes under the control of the inducible promoter *P_{grac100}*. Therefore, the *B. subtilis* expression vector pHT253
10 (MoBiTec GmbH, Göttingen, Germany) was used as backbone. The open reading frame of the *E. coli lacY* gene (Gen Bank accession number: NP_414877.1) was amplified by PCR from chromosomal DNA. The open reading frame of the gene *wbgL*, encoding for a *E. coli* O126 α-1,2-fucosyltransferases, was codon-optimized for expression in *B. subtilis* and prepared synthetically by GenScript Corp. Also, the
15 open reading frames of the *E. coli* genes *gmd* (Gen Bank accession number: NP_416557.1; encoding for a GDP-mannose-4,6-dehydratase) and *wcaG* (Gen Bank accession number: NP_416556.1; encoding for a GDP-fucose synthase) were codon-optimized for expression in *B. subtilis* and prepared synthetically by
20 GenScript Corp. Each gene enclosed in the inducible expression cassette was linked to a *B. subtilis* RBS sequence. Additionally, a suitable *B. subtilis* terminator sequence from the iGem part repository (sequence ID: BBa_B0015) was introduced in the final expression plasmid <pHT253 *P_{grac100}-wbgL-gmd-wcaG-lacY-terminator*> (SEQ ID NO: 2). The <P43-*manCBA*> integration strain was transformed with this
25 expression plasmid (SEQ ID NO: 2) by its natural competence. Gene expression was confirmed by targeted proteomics and/or by real-time PCR. Transformants were cultivated under conditions that are permissive for the *B. subtilis* to produce 2'-fucosyllactose in the presence of exogenous lactose.

Example 3: Construction of a *Bacillus subtilis* production strain for 2'-fucosyllactose

Strain A, which is described in example 2, was used as parent strain. For the production of 2'-fucosyllactose from lactose and GDP-mannose, additionally, the *E. coli* genes *lacY*, *gmd*, *wcaG* and *wbgL* were integrated in the endogenous *amyE* (5 *amyA*) locus of strain A (encoding for an alpha-amylase).

The open reading frame of the gene *wbgL* (encoding for a α -1,2-fucosyltransferase) was codon-optimized for expression in *B. subtilis* and prepared synthetically by GenScript Corp. Also, the open reading frames of the genes *gmd* (Gen Bank 10 accession number: NP_416557.1; encoding for a GDP-mannose-4,6-dehydratase) and *wcaG* (Gen Bank accession number: NP_416556.1; encoding for a GDP-fucose synthase) were codon-optimized for expression in *B. subtilis* (carried out by GenScript Corp), placed under the control of the strong constitutive *B. subtilis* promoter P_{lepA} as a two-gene operon and prepared synthetically by GenScript Corp. 15 The open reading frame of the *E. coli lacY* gene (Gen Bank accession number: NP_414877.1), encoding a lactose permease, was amplified by PCR from chromosomal DNA.

All necessary genes for the production of 2'-fucosyllactose from lactose and GDP-mannose were assembled to the constitutive expression cassette <P43-*wbgL-lacY-* 20 P_{lepA} -*gmd-wcaG*> in which each gene was linked to a *B. subtilis* RBS sequence. Additionally, a suitable *B. subtilis* terminator sequence from the iGem part repository (sequence ID: BBa_B0015) was placed downstream of this expression cassette. The above-described construct was cloned into pBR322 (New England Biolabs GmbH, Frankfurt, Germany) to generate <pBR322 flank *amyE* up-*lox71- aad9-* 25 *lox66*-P43-*wbgL-lacY-P_{lepA}*-*gmd wcaG-terminator*-flank *amyE* down> (SEQ ID NO: 3).

The resulting *amyE* integration vector (SEQ ID NO: 3) was used to transform *B. subtilis* by its natural competence. Transformants were selected by spectinomycin (100 $\mu\text{g ml}^{-1}$). Integration of the expression cassette <P43-*wbgL-lacY-P_{lepA}*-*gmd* 30 *wcaG-terminator*> into the *amyE* locus of the *B. subtilis* genome was verified by

colony PCR. Gene expression was confirmed by targeted proteomics and/or by real-time PCR.

Example 4: Construction of a *Bacillus subtilis* production strain for 2'-fucosyllactose

- 5 For the production of 2'-fucosyllactose from GDP-fucose and exogenous lactose, initially, the *E. coli* gene *lacY* was integrated into the endogenous *amyE* (*amyA*) locus (encoding for an alpha-amylase). The open reading frame of the *E. coli lacY* gene (Gen Bank accession number: NP_414877.1), encoding a lactose permease, was amplified by PCR from chromosomal DNA. The integration cassette <flank
10 *amyE* up-*lox71*- *aad9-lox66*-P43-*lacY*-flank *amyE* down > (SEQ ID NO: 4) was constructed and cloned into pBR322 (New England Biolabs GmbH, Frankfurt, Germany). *B. subtilis* was transformed with the resulting plasmid by its natural competence. Integration of the expression cassette <P43-*lacY*> into the *amyE* locus of the *B. subtilis* genome, yielding strain B, was verified by colony PCR. Gene
15 expression was confirmed by targeted proteomics and/or by real-time PCR.

- For synthesis of GDP-fucose from exogenous L-fucose a *B. subtilis* expression plasmid was constructed, comprising all necessary genes. Plasmid pDW1 was used as backbone. This plasmid was prepared synthetically by GenScript Corp and contains the genes *bla* and *erm* for selection in *E. coli* and *B. subtilis*, respectively.
20 Additionally, it contains the replicon of pMB1 for propagation in *E. coli* and the pUB110 replicon for propagation in *B. subtilis*.

- The *B. fragilis* gene *fkp* (GenBank accession number AY849806) encodes for a bifunctional fucose kinase/L-fucose-1-phosphate guanylyltransferase, the *E. coli* gene *wbgL* encodes for a α -1,2-fucosyltransferases and the *E. coli* gene *fucP* (Gen
25 Bank accession number: NP_417281.1) encodes for a L-fucose permease. The open reading frames of these genes were each codon-optimized for expression in *B. subtilis* and prepared synthetically by GenScript Corp. An expression cassette was constructed that comprises the genes *fkp*, *wbgL* and *fucP* under the control of the strong constitutive *B. subtilis* promoter P43.

Each gene enclosed in the expression cassette was linked to a *B. subtilis* RBS sequence. Additionally, a suitable *B. subtilis* terminator sequence from the iGem part repository (sequence ID: BBa_B0015) was introduced in the final expression plasmid <pDW1-P43-*fkp-wbgL-fucP-terminator*> (SEQ ID NO: 5). Strain B was
5 transformed with this expression plasmid (SEQ ID NO: 5) by its natural competence. Gene expression was confirmed by targeted proteomics and/or by real-time PCR. Transformants were cultivated under conditions that are permissive for *B. subtilis* to produce 2'-fucosyllactose in the presence of exogenous lactose and L-fucose.

10 **Example 5: Production of 2'-fucosyllactose using metabolically engineered *Bacillus subtilis* strains**

A preculture was inoculated with a metabolically engineered *Bacillus subtilis* strain suitable for the biosynthesis of 2'-fucosyllactose (as described in example 2, 3 and 4, respectively).

The preculture was incubated at 30-37 °C over night and then diluted to a starting
15 OD₆₀₀ of about 0.1 in fresh main culture medium. When the main culture reached an OD₆₀₀ of approximately 0.5, 2 mM lactose was added to the growth medium. When the inducible promoter P_{grac100} was used for gene expression, induction was carried out with lactose (2 mM) or with both lactose (2 mM) and IPTG (1 mM). For the salvage biosynthesis of GDP-fucose, additionally, 2 mM L-fucose were added.
20 Cultivation was discontinued after 24 h / 48 h and intracellular and extracellular 2'-fucosyllactose was analyzed by thin layer chromatography and/or HPLC and/or mass spectrometry (as described in WO 2017/042382 A or WO 2019/008133 A). Biosynthesis of substantial amounts of 2'-fucosyllactose was detected.